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Phylogeny of subterranean ground beetles (genus *Trechus*, Coleoptera, Carabidae) from continental Portugal

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Abstract

Understanding the composition and functioning of subterranean habitats is particularly critical, as these ecosystems host a rich array of specialized, often endemic species that remain among the least explored and protected globally. We investigated the evolutionary history of endemic troglobiont beetles of the genus *Trechus* Clairville, 1806, from continental Portugal, that have been recently red listed as Critically Endangered and Endangered of extinction. We sequenced the mitochondrial *cox1* gene of three species with restricted distributions: *T. gamae* Reboleira & Serrano, 2009, *T. lunai* Reboleira & Serrano, 2009, and *T. tatai* Reboleira & Ortuño, 2010. Our results show low genetic divergence among them and their hypothesized epigean common ancestor, *T. fulvus* Dejean, 1831. Despite this, consistent morphological differences, including reduced eyes and distinctive aedeagus shape, suggest divergent evolutionary trajectories and adaptations towards subterranean lifestyle. The unexpected lack of molecular differentiation points to a complex evolutionary history for this group of species. Future research should integrate additional molecular markers and expand taxonomic and geographic sampling, as this approach has the potential to enhance the identification of precise conservation units and, if necessary, improve existing strategies.

Keywords

Caves, molecular phylogeny, subterranean evolution, Trechini, troglobiont

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Introduction

Subterranean habitats are model systems for investigating evolutionary processes as allopatric speciation and morphological convergence (Juan et al. 2010; Pérez-Moreno et al. 2017, Mas-Peinado et al. 2022, Saclier et al. 2023). The isolation of subterranean populations, imposed by geological and hydrological barriers, leads to reduced gene flow and pronounced genetic divergence (Barr and Holsinger 1985; Juan et al. 2010; Mas-Peinado et al. 2022). In addition, adaptations to underground habitats, known as troglomorphisms (Christiansen, 1962), such as reduced eyes and wings, depigmentation, and elongated appendages have appeared independently in unrelated taxa due to the harsh conditions of total darkness and spatial fragmentation, further limiting dispersal (Jeffery 2009; Faille et al. 2010; Saclier et al. 2023).

The evolution and distribution of subterranean fauna are tightly influenced by geological, topographical, hydrological conditions, and paleogeographic context (Juan et al. 2010). Habitat fragmentation, often arising from these processes, has promoted allopatric speciation by isolating populations, breaking the genetic flow, and facilitating divergent evolutionary pathways (Mas-Peinado et al. 2022). The geological complexity of the Iberian Peninsula, shaped by prolonged tectonic activity, erosion, sedimentation, and sea-level changes has produced a mosaic of isolated karst systems, providing suitable conditions for speciation (Loidi 2017; Quesada and Oliveira 2020; Perea et al. 2021). These fragmented landscapes particularly favor taxa characterized by limited dispersal ability, such as ground beetles of the tribe Trechini Bonelli, 1810 (Faille 2019; Balart-García et al. 2023).

The non-monophyletic genus *Trechus* Clairville, 1806 is the most species-rich in the tribe Trechini, with nearly 1,000 species (Faille et al. 2014; Ortuño et al. 2017; Donabauer 2019; Möst et al. 2020). The genus *Trechus* is distributed throughout the Holarctic region and the high mountains of Africa, in both surface and underground habitats. It generally inhabits dark, damp habitats such as forest leaf litter, under stones, or near snow in high-altitude areas (Contreras-Díaz et al. 2007; Reboleira et al. 2010; Faille et al. 2013, 2014, 2023b; Möst et al. 2020). A total of 37 species and five subspecies of hypogean *Trechus* have been recorded to date on the Iberian Peninsula (Reboleira et al. 2009, 2010; Salgado et al. 2022; Sendra 2023).

Species-level diagnostic characters are largely based on morphological characteristics and the morphology of the aedeagus, and exhibit considerable intraspecific variability, presumably due to limited gene flow and the existence of small and isolated populations (Jeannel 1920, 1926, 1927, 1928; Reboleira et al. 2010; Faille et al. 2013, 2014; Möst et al. 2020; Ober et al. 2022).

The colonization of subterranean habitats by *Trechus* beetles is widely interpreted as the result of multiple independent speciation events that occurred in various regions (Contreras-Díaz et al. 2007; Faille et al. 2013, 2014; Fresneda et al. 2015, 2019). The *Trechus fulvus*-group, defined by Jeannel (1927), constitutes a monophyletic clade within the genus *Trechus* and includes 33 species distributed along the Atlantic coast of Northern Europe, the Iberian Peninsula, and North Africa (Jeannel 1920; Reboleira et al. 2009; Faille et al. 2014). This group is particularly notable for its extensive adaptive

radiation into subterranean habitats (Jeannel 1920, 1927; Reboleira et al. 2009, 2010; Faille et al. 2013, 2014; Ortuño et al. 2017). Its epigean representatives extend as far North as Norway, while the subterranean forms are mainly concentrated in southern regions characterized by lower humidity, for example, the Iberian Peninsula and North Africa (Jeannel 1920, 1941; Reboleira et al. 2009, 2010; Faille et al. 2014; Ortuño et al. 2017). Within this lineage, a high degree of geographic isolation between subterranean populations has promoted allopatric speciation and the evolution of endemic species with limited ranges (Reboleira et al. 2009, 2010; Faille 2019; Ribera et al. 2019; Ortuño et al. 2023). In southern Spain and North Africa, the *T. fulvus*-group exhibit a pattern of multiple independent colonization events originating from a widely distributed surface ancestor, *T. fulvus*, which subsequently gave rise to distinct subterranean lineages (Faille et al. 2014). These distinct evolutionary trajectories are consistent with the climate-relict hypothesis, which proposes that Miocene climate aridification played a key role in the resulting underground specialization and isolation of beetle populations (Jeannel 1943; Juan et al. 2010; Faille et al. 2014). *Trechus fulvus* Dejean, 1831 stands out for its ecological versatility and extensive geographic range, broadly distributed across Western Europe and North Africa, and its notable adaptability to both surface and subterranean habitats (Reboleira et al. 2009, 2010; Faille et al. 2014; Ortuño et al. 2017; Ribera et al. 2019). In the Iberian Peninsula, it occurs in caves and in the mesovoid shallow substratum (MSS), a shallow subterranean habitat composed of a complex network of cracks and small, air-filled voids within rocky substrates (Juberthie et al. 1980; Mammola et al. 2016; Eusébio et al. 2021, 2023). Four hypogean species of *Trechus* are present in mainland Portugal, all located in the central karst massif: *T. machadoi* Jeannel, 1941, *T. gamae* Reboleira & Serrano, 2009, and *T. lunai* Reboleira & Serrano, 2009, which are exclusively found in caves and MSS of different sub-units of the Estremenho karst massif, and *T. tatai* Reboleira & Ortuño, 2011, restricted to the Montejunto karst massif, roughly 40 km Southwest of the Estremenho massif (Reboleira et al. 2009, 2010; Reboleira and Eusébio 2021; Boieiro et al. 2023). The aim of this study was to investigate the evolutionary history of these three endemic troglobiont species of *Trechus* from mainland Portugal, using mitochondrial cox1 sequences to assess its interspecific genetic diversity and reconstruct phylogenetic relationships, and to understand whether they form a monophyletic group derived from the widespread epigean species *T. fulvus*.

Methods

Sampling

Specimens of *Trechus* were obtained from both entomological collections and field sampling (Suppl. material 1: table S1). The collection material was accessed from the Subterranean Ecology Group at the Centre for Ecology, Evolution, and Environmental Change (CE3C) in Portugal and from the Staatliches Museum für Naturkunde Stuttgart (SMNS) in Germany. These repositories included material from Portugal, Spain,

France, and Morocco. Field sampling focused on the Lusitanian group, which includes *T. machadoi*, *T. gamae*, *T. lunai*, and *T. tatai*, in the known habitats of these species. Pitfall traps were used according to the methodologies of Reboleira et al. (2009) for caves and Eusébio et al. (2021) for MSS habitats. Pitfall traps were baited with pig liver and partially filled with 1,2-propanediol (Sigma-Aldrich, purity ≥ 99%), as preservative liquid. Field samples were then preserved in 96% ethanol and stored at 4 °C until further processing.

DNA extraction, PCR amplification and sequencing

The DNA extractions were performed using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Prior to extraction, precise incisions were made in the thorax and head membranes of each specimen under a Leica MZ6 Microscope (Leica Microsystems) (Faille et al. 2014, 2023a). These non-destructive methods ensured the preservation of specimens for subsequent morphological examination, including the detailed analysis of the aedeagus (Faille et al. 2010). Overnight incubation for DNA extraction was conducted using the HCL Heating-ThermoMixer MHL 23 (Ditabis, Pforzheim, Germany), maintained at 56 °C with a rotational speed of 300 revolutions per minute, during which the specimens were fully immersed in 180 µL of Buffer ATL and 20 µL of Proteinase K. After DNA extraction, 50 µL of the genetic material was set aside for immediate analysis, while 150 µL was cryopreserved at -20 °C for future research. All DNA samples are stored at the SMNS, Stuttgart, Germany.

For Polymerase Chain Reaction (PCR) amplification, the mitochondrial fragment cytochrome c oxidase sub-unit I (COI or *cox1*) was sequenced. This fragment was selected due to its high copy number per cell, the availability of well-established primer sequences, and its relatively low recombination rate (Mueller 2006; Goodall-Copestake et al. 2012; Bogale et al. 2020). The PCR amplification procedure involved the preparation of a Master Mix for each DNA sample, combining water, Master Mix Tracer, and target-specific primers (Table 1). The PCR reactions were conducted in a SensoQuest Labcycler Thermocycler (SensoQuest, Germany).

After amplification, the PCR products were visualized by electrophoresis on a 1% agarose gel (1:100 agarose / TAE Buffer 1×) stained with ROTI-®GelStain (Carl Roth). Electrophoresis was performed in a Mupid One Electrophoresis System (Mupid CO. LTD., Tokyo, Japan) for 20 minutes at 100 V. Gel images were subsequently captured using Gel Doc XR+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The resulting products were sent for sequencing to the Macrogen Europe Company in Amsterdam, Netherlands.

Table 1. Molecular marker, primers (F - Forward; R - Reverse) and corresponding sequences.

Marker	Primer	Sequence	Reference
cox1	Jerry (F)	5' CAACATTTATTTTGATTTTTTGG 3'	Simon et al. 1994
	Pat (R)	5' TCCA(A)TGCACTAATCTGCCATATTA 3'	

Phylogenetic analysis

The obtained *cox1* DNA sequences were assembled and edited using BioEdit (v. 7.2) (Hall 1999) and Geneious Prime (v. 2024.0.1) (https://www.geneious.com) software. Subsequently, multiple sequence alignment was performed utilizing the MAFFT online version 7.0 software (Katoh et al. 2019) with default parameters.

ModelTest-NG (Darriba et al. 2020), integrated within RAxML GUI version 2.0.10 (Edler et al. 2021), was used to perform model selection. The TPM1uf+I+G4 substitution model was selected, and maximum likelihood analyses were conducted using RAxML GUI 2.0.10 (Edler et al. 2021). Ten independent runs were implemented to search for the best-scoring tree, and the reliability of the inferred tree was evaluated using 200 bootstrap replications. To root the phylogenetic tree, *Trechus obtusus* Erichson, 1837 was chosen as the outgroup, following the approach of Faille et al. (2014) and Wooden and Caterino (2024). The species, that belongs to the *T. quadristriatus*-group (Ortuño and Arillo 2005), was selected due to its close evolutionary relationship with the *T. fulvus*-group (Faille et al. 2013, 2014).

The phylogenetic tree was visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree), with annotations created in Interactive Tree of Life (iTOL) (v. 6) (Letunic and Bork 2024). The DNA sequences were deposited in the GenBank database, with specific accession numbers provided in Suppl. material 1: table S2.

Morphological analyses

Morphological assessment involved high-resolution photographic documentation of *Trechus* specimens, capturing detailed images of the habitus, ocular area, and male aedeagi. The body structures were photographed with a Canon EOS 5DS R camera (Canon, Tokyo, Japan). The photographs of the eyes and aedeagi were acquired using a self-constructed focus stacking photography system equipped with a Sony a7RII camera (Sony Corporation, Minato, Tokyo, Japan) and a Mitutoyo M Plan Apo 20x microscope objective (Mitutoyo Corporation, Kawasaki, Tokyo, Japan). Post-processing procedures were executed using Adobe Photoshop CS6 (v. 13.0.1) (Adobe Systems Inc., San José, California, USA). Image stacking and focus merging processes were conducted in the software Helicon Focus (v. 8.1.0) (Helicon Soft Ltd., Kharkov, Ukraine).

Following image acquisition, three fundamental morphological traits: body length; pronotum width; and eye dimensions, were measured with ImageJ software (http://rsb.info.nih.gov/ij). The body length was measured from the apex of the elytra to the middle of the labrum, and pronotum width was measured at its maximum breadth, according to Reboleira et al. (2009). The curvature of the pronotum was also visually assessed due to its taxonomic significance, as described by Jeannel (1927). To evaluate the ocular regression within the *T. fulvus*-group, the maximum eye width was measured as an indicator of potential convergent evolutions in visual acuity across all specimens. Eye width was measured from the basal margin to the apical margin, along the central axis of the eye, in accordance with Reboleira et al. (2009). In addition,

a detailed comparative morphological analysis of the aedeagus was performed, examining the shapes of the outlines, basal margin and apex, the number of setae on the parameres, and the structures of the endophallus.

Statistical analysis

All statistical analyses were performed in R statistical environment (v. 4.4.1) (R Core Team 2024) using RStudio (v. 2024.04.2+764 for Windows). To assess morphological variation, specimens were classified according to their biotope type, defined based on their environmental conditions and their potential impact on the evolutionary processes of the species: (1) Epigean lifestyle: *T. lallemantii* and *T. fulvus* found in surface habitats, where species are exposed to more variable environmental conditions, including light, temperature, and humidity; (2) Epigean species with a subterranean lifestyle: *T. fulvus* from subterranean habitats, such as cave entrances and areas where the species interacts with both subterranean and surface conditions; (3) Subterranean lifestyle: *T. gamae*, *T. lunai*, and *T. tatai* collected from deep cave zones, marked by permanent darkness, stable temperatures, and high humidity.

Data visualization was performed using the "ggplot2" package (Wickham 2016). Afterwards, a Shapiro-Wilk test was applied to assess the normality of data distributions, and a Levene's test was employed to evaluate the homogeneity of variances, using the "car" package (Fox and Weisberg 2018). Upon verification of the assumptions of normality and homoscedasticity, a one-way analysis of variance (ANOVA) was conducted to test for statistically significant differences in morphological features among biotope categories. When significant differences were detected, a Tukey's Honestly Significant Difference (HSD) post hoc test was applied to determine pairwise differences between groups.

Phylogenetic analysis

A maximum likelihood (ML) phylogenetic tree was constructed to elucidate the evolutionary relationships among *Trechus* species, with an emphasis on hypogean taxa from continental Portugal. Branch lengths represent nucleotide substitutions per site, as indicated by the scale bar (0.01 substitutions/site). *Trechus machadoi* was excluded from the analyses due to a lack of newly collected specimens from the type locality.

Results

The resulting tree reveals clear genetic structuring correlated with geographic distribution, with well-defined clusters of *Trechus* specimens from Madeira Island (yellow), Morocco (orange), France (blue), and the Iberian Peninsula (red and green) (Fig. 1).

Clade B, representing the *T. fulvus*-group, is divided into two well-supported subclades. Clade C comprises only *T. lallemantii* from northern Morocco (bootstrap = 95%), while clade D comprises all other species in the group (bootstrap = 100%).

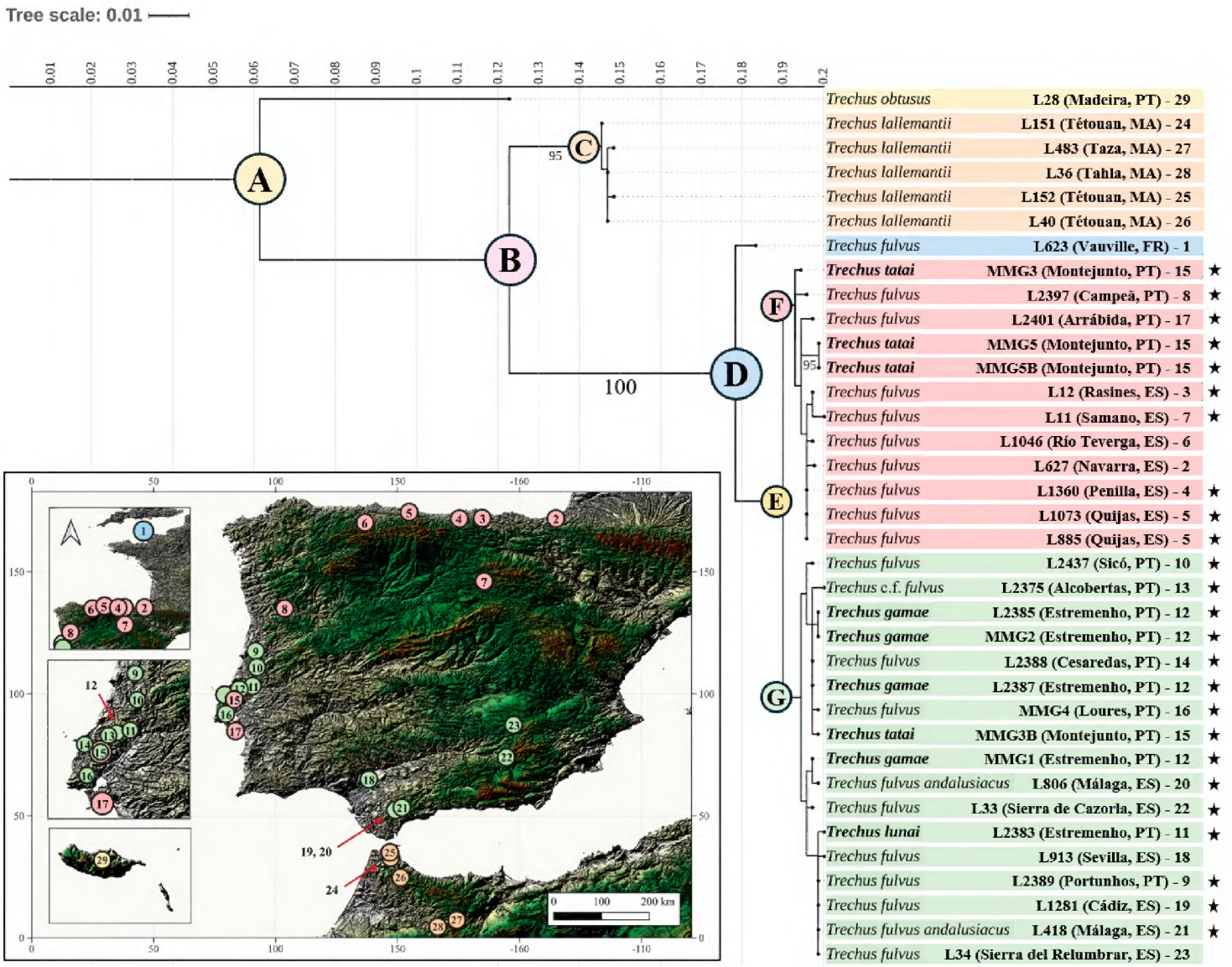
Tree scale: 0.01
0.01 0.02 0.03 0.04 0.05 0.06 0.07 0.08 0.09 0.1 0.11 0.12 0.13 0.14 0.15 0.16 0.17 0.18 0.19 0.2
A
B
C
D
E
F
G
95
100
95
Trechus obtusus L28 (Madeira, PT) - 29
Trechus lallemantii L151 (Tétouan, MA) - 24
Trechus lallemantii L483 (Taza, MA) - 27
Trechus lallemantii L36 (Tahla, MA) - 28
Trechus lallemantii L152 (Tétouan, MA) - 25
Trechus lallemantii L40 (Tétouan, MA) - 26
Trechus fulvus L623 (Vauville, FR) - 1
Trechus tatai MMG3 (Montejunto, PT) - 15 ★
Trechus fulvus L2397 (Campeã, PT) - 8 ★
Trechus fulvus L2401 (Arrábida, PT) - 17 ★
Trechus tatai MMG5 (Montejunto, PT) - 15 ★
Trechus tatai MMG5B (Montejunto, PT) - 15 ★
Trechus fulvus L12 (Rasines, ES) - 3 ★
Trechus fulvus L11 (Samano, ES) - 7 ★
Trechus fulvus L1046 (Río Teverga, ES) - 6
Trechus fulvus L627 (Navarra, ES) - 2
Trechus fulvus L1360 (Penilla, ES) - 4 ★
Trechus fulvus L1073 (Quijas, ES) - 5 ★
Trechus fulvus L885 (Quijas, ES) - 5 ★
Trechus fulvus L2437 (Sicó, PT) - 10 ★
Trechus c.f. fulvus L2375 (Alcobertas, PT) - 13 ★
Trechus gamae L2385 (Estremenho, PT) - 12 ★
Trechus gamae MMG2 (Estremenho, PT) - 12 ★
Trechus fulvus L2388 (Cesaredas, PT) - 14 ★
Trechus gamae L2387 (Estremenho, PT) - 12 ★
Trechus fulvus MMG4 (Loures, PT) - 16 ★
Trechus tatai MMG3B (Montejunto, PT) - 15 ★
Trechus gamae MMG1 (Estremenho, PT) - 12 ★
Trechus fulvus andalusiacus L806 (Málaga, ES) - 20 ★
Trechus fulvus L33 (Sierra de Cazorla, ES) - 22 ★
Trechus lunai L2383 (Estremenho, PT) - 11 ★
Trechus fulvus L913 (Sevilla, ES) - 18
Trechus fulvus L2389 (Portunhos, PT) - 9 ★
Trechus fulvus L1281 (Cádiz, ES) - 19 ★
Trechus fulvus andalusiacus L418 (Málaga, ES) - 21 ★
Trechus fulvus L34 (Sierra del Relumbrar, ES) - 23
19, 20
24
0 100 200 km

Figure 1. Maximum likelihood phylogenetic tree of *Trechus* beetles inferred from *cox1* gene sequences. Bootstrap values (≥ 70%) are shown at the respective nodes. The clades include **A** the outgroup *T. obtusus* from Madeira, Portugal **B** the divergence between *T. lallemantii* and the *T. fulvus*-clade **C** *T. lallemantii* from northern Morocco **D** *T. fulvus* from France and the Iberian *Trechus* species **E** Iberian species of *Trechus*; and two poorly supported clades **F** *T. fulvus* from the Iberian Peninsula and *T. tatai* **G** Iberian *T. fulvus* and the hypogean species *T. gamae*, *T. lunai*, and *T. tatai* species. Subterranean species are marked with an asterisk. The studied specimens are color-coded to correspond to their positions on the phylogenetic tree. The geographical distribution and specific locations (1–29) corresponding to the phylogenetic analysis are annotated. Locations are abbreviated using ISO 3166-1 alpha-2 codes: MA (Morocco), PT (Portugal), ES (Spain), and FR (France). Tree scale: 0.01 substitutions/site.

Within clade D, *T. fulvus* specimen from northern France form a distinct lineage relative to Iberian specimens, which are grouped in clade E. This Iberian clade is subdivided into clades F (bootstrap = 54%) and G (bootstrap = 62%). Clade F includes Iberian *T. fulvus* and three *T. tatai* specimens, without clear phylogenetic structure. Clade G contains southern Iberian *T. fulvus* as well as the hypogean species *T. gamae*, *T. lunai*, and a single specimen of *T. tatai*. The relationships between members of clade G are also weakly supported, with bootstrap values < 63%.

Based solely on the mitochondrial *cox1* gene, Portuguese subterranean species of *Trechus* are phylogenetically nested within the larger *T. fulvus* clade of the Iberian Peninsula (clades F and G). Despite their distinct troglomorphic adaptations and current

taxonomic classification, these species do not form a monophyletic group but are instead scattered within the clade of *T. fulvus* (clade E). However, the low bootstrap support values for clades F and G indicate unresolved phylogenetic relationships.

Morphological analysis

Morphometric analyses were performed on 28 specimens of the *T. fulvus*-group, representing five species (namely *T. fulvus*, *T. gamae*, *T. lunai*, *T. tatai*, *T. lallemantii*) for interspecific variation, and one subspecies (*T. f. andalusiacus*) for intraspecific variation. Measurements included body length, pronotum width, and eye dimensions (Suppl. material 1: table S3).

No statistically significant differences were observed in body length between biotopes ($p = 0.149$, ANOVA) (Suppl. material 1: table S4, fig. S2). In contrast, significant differences were detected between biotopes in terms of pronotum width ($p = 0.015$, ANOVA) (Suppl. material 1: table S5, fig. S3) and eye width ($p = 1.07 \times 10^{-9}$, ANOVA) (Suppl. material 1: table S6, fig. S4).

Post-hoc Tukey's HSD tests revealed significant differences in pronotum width between *T. lallemantii* and Portuguese hypogeansubterranean species (adjusted $p = 0.0491$) and between *T. fulvus* and hypogean species (adjusted $p = 0.0248$) (Suppl. material 1: table S7). No significant difference was detected between *T. lallemantii* and *T. fulvus* (adjusted $p = 0.8731$). Regarding eye width, subterranean species from mainland Portugal had significantly smaller eyes than those of *T. fulvus* and *T. lallemantii* (adjusted $p < 0.0001$; Fig. 2). A smaller but significant difference was also observed between *T. fulvus* and *T. lallemantii* in terms of eye width (adjusted $p = 0.0293$).

Individuals with significantly reduced eye width (< 0.20 mm) included *T. gamae*, *T. lunai*, and *T. tatai*, all grouped in clades F and G. No other taxa analyzed had eye dimensions below this threshold.

Morphological analysis of the aedeagus in male specimens of *T. fulvus*, *T. lallemantii*, *T. gamae*, *T. lunai*, and *T. tatai* revealed distinct morphological characteristics specific to each species (Fig. 3). *T. machadoi* was excluded due to the absence of male specimens. The Portuguese continental hypogean species – *T. gamae* (Fig. 3C), *T. lunai* (Fig. 3D), and *T. tatai* (Fig. 3E) - exhibited distinctive features consistent with their species description and geographical isolation. All species exhibit unique shapes of the basal margin and apex, a distinct number of paramere setae, and unique endophallus structures. These features were consistently observed in all specimens and did not overlap between species. *T. lallemantii* (Fig. 3A) and *T. fulvus* (Fig. 3B) have a different aedeagus morphology from that of Portuguese troglobionts.

Discussion

Our phylogenetic analysis based on the mitochondrial *cox1* gene indicates that the Portuguese hypogean species – *Trechus gamae*, *T. lunai*, and *T. tatai* - are genetically clustered with populations of *T. fulvus* from both surface and underground habitats across the

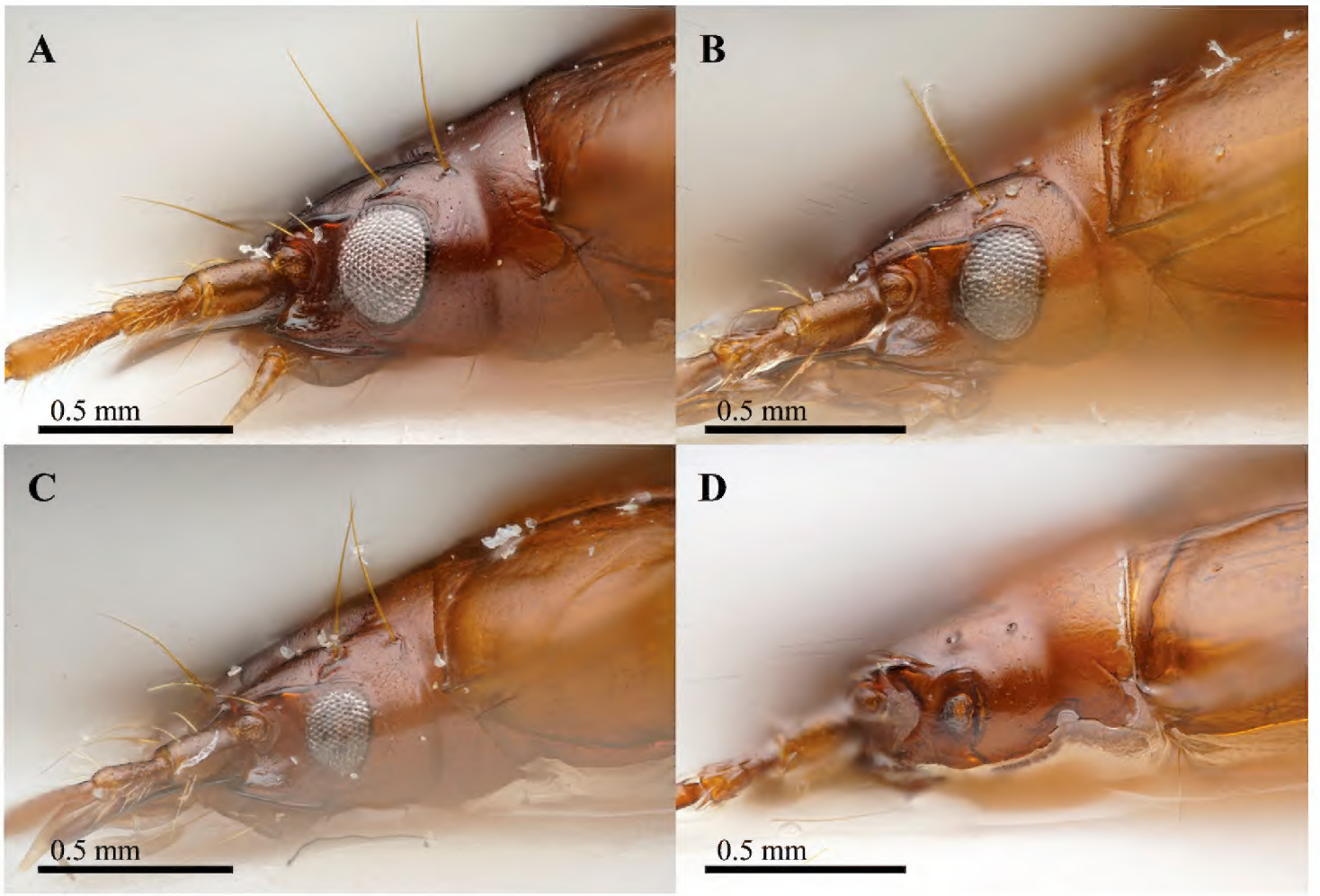

Figure 2. Comparative morphology of eye size in the *Trechus* specimens studied **A** *T. lallemantii* (L483) from a surface habitat in Morocco **B** *T. fulvus* (L623) from a surface habitat in France **C** *T. fulvus* (L33) from an underground habitat in Spain, and **D** *T. tatai* (MMG5B) from an underground habitat in Portugal.

Iberian Peninsula, suggesting low genetic divergence between these taxa. This Iberian clade is phylogenetically distinct from *T. fulvus* specimens from northern France, in agreement with Faille et al. (2014). Furthermore, *T. lallemantii* forms a well-supported clade, distinct from the Iberian group. Nevertheless, the phylogenetic tree lacks resolution, with most clades supported by bootstrap values below 70%. In contrast, morphological analyses reveal clear phenotypic differences, particularly in the morphology of the aedeagus.

Portuguese hypogean species of the genus *Trechus* are confined to small, geographically isolated karst massifs (Jeannel 1941; Reboleira et al. 2009, 2010). Our phylogenetic analyses based on the *cox1* gene show that Lusitanian hypogean *Trechus* are nested within a larger *T. fulvus* clade that includes both epigean and subterranean populations distributed throughout the Iberian Peninsula. This phylogenetic pattern could reflect a recent colonization of underground habitats, like the slight divergence observed in *Trechus* species from caves in the Canary Islands (Contreras-Díaz et al. 2007), or it could indicate historical mitochondrial introgression between these populations (Kosuda et al. 2016). However, the low support values associated with clades F and G (<70%) demonstrate that the relationships between these taxa are tentative and require further phylogenetic refinement (Hillis and Bull 1993). In contrast, the phylogenetic position of *T. lallemantii* as a sister lineage to the *T. fulvus*-group is well supported, which is consistent with the results of Faille et al. (2014), who documented a similar pattern among *T. fulvus* populations in southern Spain.

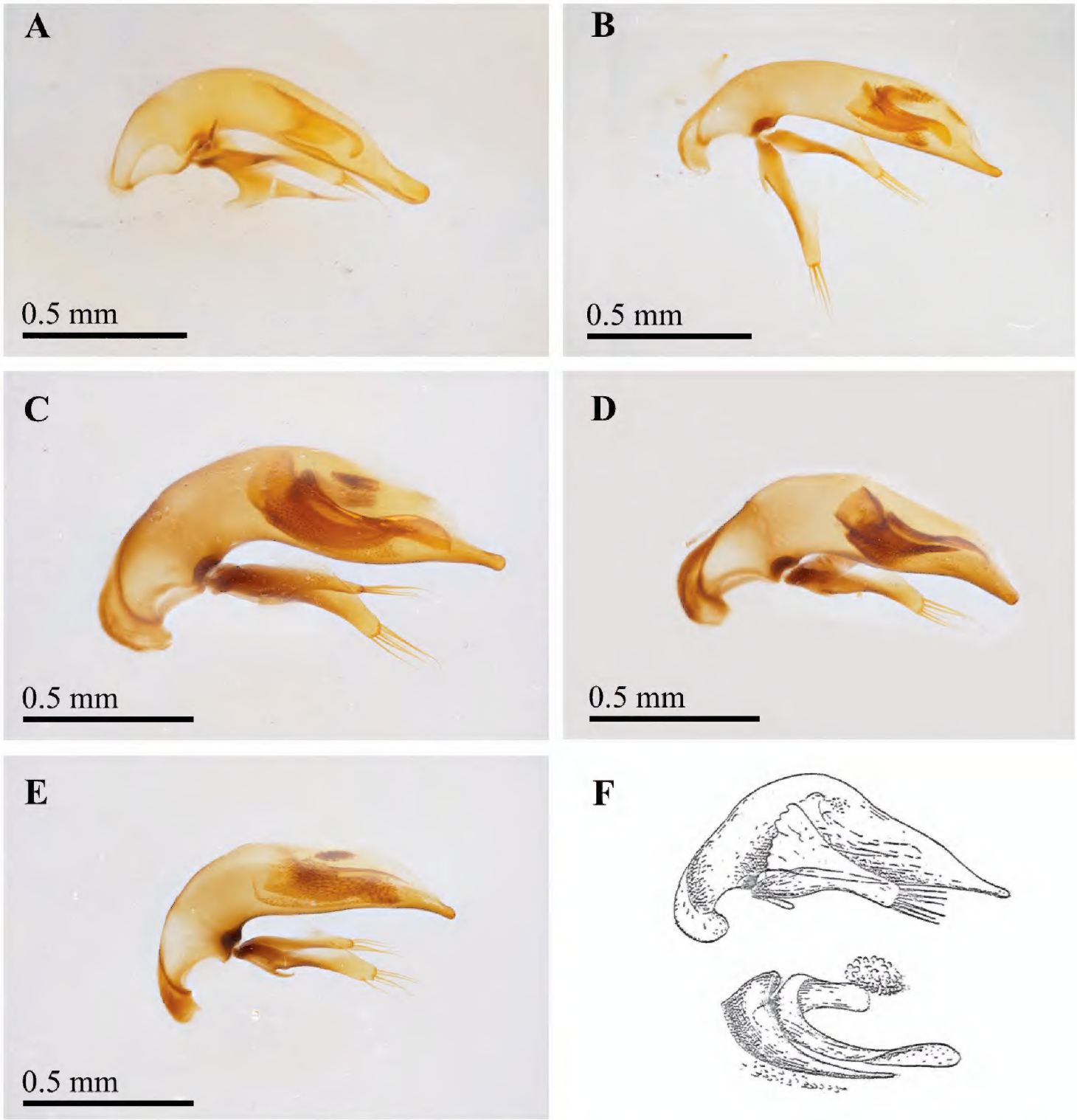

Figure 3. Comparative morphology of the aedeagus in the *Trechus fulvus*-group. **A** *T. lallemantii* (L483) **B** *T. fulvus* (L913) **C** *T. gamae* (L2387) **D** *T. lunai* (L2383); **E** *T. tatai* (MMG3), and **F** illustration of the aedeagus of *T. machadoi* from Jeannel (1941).

Trechus fulvus exhibits subtle intraspecific variation in eye development, with individuals collected from caves having smaller eyes than their surface-dwelling counterparts. This gradient, first noted by Jeannel (1920), may indicate phenotypic plasticity or incipient troglomorphic evolution (Trontelj 2019; Wynne 2022). Although less pronounced than in hypogean species, these reductions nevertheless suggest adaptive responses to underground habitats (Christiansen 1962; Trontelj 2019). Troglobiont species – *T. gamae*, *T. lunai*, and *T. tatai* – have significantly smaller eyes than their epigean counterparts, reflecting characteristics observed in other hypogean species of Trechini (Faille 2019; Pavićević et al. 2020; Ober et al. 2022). The morphological

divergence observed within the *T. fulvus*-group supports the hypothesis of convergent evolution driven by adaptation to underground life, particularly regarding the reduction of eyes, a classical troglomorphic trait (Jeannel 1926, 1941; Christiansen 1962; Poulson and White 1969; Reboleira et al. 2009, 2010; Faille 2019; Jeffery 2020; Balart-García et al. 2023). The recurrence of eye reduction in phylogenetically distinct *Trechus* species further supports the hypothesis of multiple independent colonization events in subterranean habitats (Faille et al. 2014). This convergence, observed in different *Trechus* lineages, is probably the result of similar selective pressures in underground habitats, such as permanent darkness, , and is reinforced by the structure of subterranean habitats, particularly the presence or absence of cracks and voids, which mediate connectivity between populations (Reboleira et al. 2009, 2010; Faille et al. 2015). Comparable patterns have also been observed in other lineages of cave-dwelling invertebrates (e.g. Ribera et al. 2019; Ober et al. 2022).

The distinctive morphologies of the aedeagus in *T. gamae*, *T. lunai*, and *T. tatai* are consistent with previous taxonomic descriptions (Reboleira et al. 2009, 2010). The stability of these morphological features reflects the geographical isolation of the populations, which is influenced primarily by discontinuities in their subterranean habitats, (Reboleira et al. 2009, 2010; Reboleira 2012). Discontinuities in suitable underground habitats are likely to limit gene flow and promote morphological divergence through allopatric processes (Reboleira et al. 2010; Faille et al. 2015). Despite the morphological consistency of *T. tatai* with its original description (Reboleira et al. 2010), our *cox1* analysis indicated that specimens attributed to this species are distributed across two distinct clades, F and G. This genetic pattern, which is not accompanied by morphological divergence, suggests that the mitochondrial lineages observed may not represent distinct evolutionary units, which is further complicated by low bootstrap support and raises the possibility of incomplete lineage sorting or recent divergence (Hillis and Bull 1993).

The discrepancy observed between morphological differentiation and mitochondrial phylogeny is rare in the genus *Trechus* and highlights the evolutionary complexity within the *T. fulvus*-group. While morphological traits - particularly eye size and aedeagus structure – strongly suggest divergence due to subterranean adaptation and isolation, the mitochondrial *cox1* gene alone does not provide sufficient resolution to reliably delineate species boundaries. To explain these contradictory patterns, three non-mutually exclusive hypotheses can be considered. One possibility is that the Portuguese troglobiont species, currently recognized as distinct, are in fact synonyms of *Trechus fulvus*, with the observed differences simply reflecting intraspecific variability. However, this scenario is not strongly supported by current information, as morphological traits are both stable and geographically correlated, and phylogenetic support remains weak (Reboleira et al. 2010). Alternatively, mitochondrial introgression and the limitations of the *cox1* marker in determining species boundaries may play a role. Introgression, although documented in Carabidae (Kosuda et al. 2016) and widespread in insects (Ballard and Whitlock 2004; Bonnet et al. 2017; Thelwell et al. 2000; Zakharov et al. 2009; Darras and Aron 2015), is rare in *Trechus* (Fresneda et al. 2019), but could plausibly explain the lack of concordance between the molecular

and morphological datasets for *Trechus fulvus*, *T. gamae*, *T. lunai*, and *T. tatai*. The use of a multilocus approach, incorporating rapidly evolving nuclear genes (Mastrantonio et al. 2016) or even whole genome sequencing (Mardis 2017), would allow this introgression hypothesis to be tested and the relationships between species to be resolved (Faille et al. 2010, 2013, 2014; Gough et al. 2018; Del Pedraza-Marrón et al. 2019; Saclier et al. 2023; Wooden and Caterino 2024). Finally, it is also plausible that *T. fulvus* represents a complex of morphologically similar but evolutionarily distinct cryptic species (Bickford et al. 2007; Hending 2024), an interpretation supported by its ecological and morphological diversity, fragmented distribution, and localized variations (Jeannel 1920; Ortuño et al. 2017). More extensive sampling of populations in surface and underground habitats throughout the species' range, from the Iberian Peninsula (Ortuño 2004, 2008; Faille et al. 2010; Ortuño et al. 2014) or even northern Europe (Jeannel 1920), and with expanded genetic datasets, would facilitate a more definitive delimitation of species and clarify evolutionary trajectories within this group (Zwickl and Hillis 2002; Nabhan and Sarkar 2012; Che et al. 2021).

Despite these unresolved taxonomic issues, the minimal genetic divergence between *T. fulvus*, *T. gamae*, *T. lunai*, and *T. tatai* complicates species delimitation, which is an essential prerequisite for defining conservation units (Ficetola et al. 2018; Duran et al. 2024). However, immediate conservation measures remain a priority. *Trechus machadoi*, *T. gamae*, *T. lunai*, and *T. tatai* are currently listed as "Endangered" or "Critically Endangered" in the Red Book of Invertebrates of Mainland Portugal (Boieiro et al. 2023). Until further multilocus or genomic data provide better taxonomic resolution, it is prudent to maintain their current conservation status under the precautionary principle. Premature synonymization could inadvertently weaken legal protections, particularly for taxa with very restricted ranges such as *T. machadoi*, which has not been observed since its description (Reboleira and Eusébio 2021), and *T. tatai*, which is endemic to a single cave (Reboleira et al. 2010).

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Supplementary material 1

Supplementary information

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Data type: docx

Explanation note: Occurrences, morphological, phylogenetic and images.

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